

Characterization of the Oligogalacturonide-Induced Oxidative Burst in Cultured Soybean (*Glycine max*) Cells¹

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The rapid release of H₂O₂ by elicited plant cells, recently termed the oxidative burst, was investigated in suspension-cultured soybean (*Glycine max* Merr. cv Kent) cells stimulated with a purified polygalacturonic acid (PGA) elicitor. Examination of the elicited cells by fluorescence microscopy revealed that virtually every living cell participates in the elicitor-induced H₂O₂ burst. Measurement of the kinetics of the response using a macroscopic fluorescence-based assay indicated that approximately 100 molecules of H₂O₂ are generated per PGA molecule added, achieving a cumulative H₂O₂ concentration of approximately 1.2 mmol L⁻¹ of packed cells. At the height of the defense response, 3 × 10⁻¹⁴ mol of H₂O₂ cell⁻¹ min⁻¹ are produced, a value comparable to the rate of H₂O₂ production by myeloid cells of mammals. Variables affecting the rate and magnitude of the soybean oxidative burst were found to be mechanical stress, extracellular pH, and cell age. The PGA-induced oxidative burst was shown to undergo both homologous and heterologous desensitization, a characteristic of signal transduction pathways in animals. Homologous desensitization was obtained with PGA, and heterologous desensitization was observed with the G protein activator mastoparan, consistent with earlier observations showing that G proteins perform a regulatory function in this pathway. Finally, a model describing the possible role of the PGA-induced oxidative burst in the overall scheme of plant defense is proposed.

Most plant cells possess the capacity to both synthesize and degrade reactive oxygen species such as H₂O₂ and O₂⁻. Plant-derived oxidants are commonly produced during such processes as auxin stimulation (Brightman et al., 1988), mechanical distortion (Bradley et al., 1992), UV irradiation (Murphy and Huerta, 1990), cold acclimation (Okuda et al., 1991), and defense response elicitation (reviewed by Sutherland, 1991). Enzymes that degrade oxidants, such as catalases, peroxidases, and superoxide dismutases are either constitutively expressed or induced in response to stress (Montalbini and Buonauro, 1986; Buonauro et al., 1987; Zacheo and Blevé-Zacheo, 1988; Bowler et al., 1989). In many cases, neither the sources nor the functions of the oxidative components are well understood, but their synthesis in nearly all higher life forms suggests that their physiological purpose is not readily replaced or deleted.

The oxidative response that has received the broadest scrutiny in plants has been the release of H₂O₂ or O₂⁻ during

stimulation by pathogenic elicitors. Doke and co-workers reported superoxide production in potato tubers (Doke, 1983) and potato leaves (Chai and Doke, 1987) 1 to 4 h after inoculation with an incompatible race of *Phytophthora infestans*, an observation later supported by Jordan and DeVay (1990). A fungal elicitor preparation from the pathogen *Colletotrichum lindemuthianum* has also been shown to stimulate the accumulation of lipid peroxidation products in bean suspension cultures 9 h after treatment (Rogers and Anderson, 1987). Using root knot nematodes to elicit hypersensitive cell death in excised tomato roots, Zacheo and Blevé-Zacheo (1985) demonstrated an enhancement of superoxide production 3 d after tissue infestation. Moreover, the phytopathogenic bacteria *Pseudomonas syringae* has been shown to trigger superoxide release 1 to 4 h after treatment of a nonhost cell suspension (Keppler et al., 1989) and 3 to 6 h after inoculation of a tobacco plant (Adam et al., 1989).

Although not originally appreciated, the oxidative response is now known to constitute one of the earliest events following stimulation of cells with elicitors. We originally observed the release of oxidative species from cell suspensions of cotton, tobacco, and soybean only minutes after the addition of a crude extract from the pathogenic fungus *Verticillium dahliae* (Low and Heinsteins, 1986; Apostol et al., 1989a). This observation has since been confirmed in several other plant/pathogen interactions. These include soybean/*Phytophthora megasperma* f. sp. *glycinea* (Lindner et al., 1988), bean/*Colletotrichum lindemuthianum* (Anderson et al., 1991), white clover/*Pseudomonas corrugata* (Devlin and Custine, 1992), tomato/*Cladosporium fulvum* (Vera-Estrella et al., 1992), and spruce/*Amanita muscaria*, *Hebeloma crustuliniforme*, or *Heterobasidium annosum* (Schwacke and Hager, 1992).

More recently, a purified fraction from pectic hydrolysates was shown to stimulate H₂O₂ biosynthesis within seconds to minutes of its addition to soybean cells (Legendre et al., 1992). In contrast, the better-characterized defense responses involving de novo protein synthesis (e.g. phytoalexin production, hydrolase biosynthesis, proteinase inhibitor expression) all require several hours to reach their effective levels (Bell, 1981). Because of the cytotoxicity of the activated oxygen compounds and the rapidity of their expression following elicitation, the oxidative burst has been suggested to constitute the first line of defense against pathogen invasion (Kim et al., 1988; Apostol et al., 1989a; Bradley et al., 1992). Unfortunately, unlike in myeloid cells of mammals (Morel et

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Abbreviation: PGA, polygalacturonic acid.

al., 1991), there are no known inherited disorders in plants to establish the absolute requirement of the oxidative burst in resistance to infection.

In an effort to understand better the elicitor-stimulated oxidative burst in plants, we have undertaken to characterize the response quantitatively in a well-defined soybean cell-suspension culture system. Using a homogeneous PGA elicitor to stimulate the defense response, we have estimated the fraction of cells participating in the H_2O_2 burst and the number of H_2O_2 molecules generated per elicitor added and extrapolated the final concentration of H_2O_2 produced under optimal conditions in vivo during an analogous elicitation event. We have also partially characterized the down-regulation or desensitization of the pathway following its initial stimulation.

MATERIALS AND METHODS

Materials

Mastoparan, a peptide from the venom of *Vespula lewisii*, was from Sigma, and Mas17, its inactive homolog (two amino acid substitutions), was from Peninsula Laboratories. Bio-Gel P-6DG was from Bio-Rad.

Plant Cell Culture

Cell-suspension cultures of soybean (*Glycine max* Merr. cv Kent) were maintained on W-38 medium (Hasegawa et al., 1980) as described earlier (Low and Heinsteins, 1986). This medium contained 4.4 g L^{-1} of Murashige and Skoog basal salts with minimal organics (Sigma), as described by Linsmaier and Skoog (1965), 2 g L^{-1} of bacto-tryptone (Difco), 30 g L^{-1} of Suc (ICN), 3 mg L^{-1} of 2,4-D (Sigma), and 0.1 mg L^{-1} of kivetin (Sigma) at a final pH of 5.7. Cell cultures were maintained by transferring 9 cm^3 of filtered cells every 10 d to 100 mL of fresh medium. When an elicitation study was to be conducted, 6 cm^3 of filtered cells were transferred to 100 mL of fresh medium and allowed to grow for approximately 36 h before use.

Elicitor Preparation

An oligogalacturonic acid fraction (called PGA) that elicits H_2O_2 production in soybean cell suspensions was purified from an acid hydrolysate of citrus pectin (Sigma) according to the method of Nothnagel et al. (1983). It was subsequently dialyzed against distilled water using dialysis tubing with a cutoff at an M_r of approximately 500 (Spectrum) and pH adjusted to the pH (approximately 4.8) of the culture medium. The preparation used in this study contained 0.678 mg mL^{-1} of galacturonic acid equivalents, as determined by the method of Blumenkrantz and Asboe-Hansen (1973).

Observation of H_2O_2 Production by Fluorescence Microscopy

A 36-h-old cell suspension (0.5 mL) was mixed gently for 15 s with $7 \mu\text{L}$ of a 0.2-mg mL^{-1} stock solution ($2.8 \mu\text{g mL}^{-1}$ final concentration) of pyranine (Molecular Probes, Inc.) either alone or in combination with $10 \mu\text{L}$ of PGA ($13.5 \mu\text{g}$

mL^{-1}). Each sample was subsequently spotted onto a microscope slide and analyzed by fluorescence microscopy. Photographs were taken periodically, starting 30 s after PGA addition using T-MAX 3200 speed film (Kodak) with an exposure time of 12 s for all micrographs. When no picture was taken, the excitation lamp shutter was closed to prevent damage to the dye and to the cells.

Spectrofluorimetric Determination of H_2O_2 Production

H_2O_2 , the predominant oxidative species elicited in cultured soybean cells (Apostol et al., 1989a), was detected via the peroxidase-mediated oxidative quenching of the fluorescent reporter dye pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt; excitation wavelength 405 nm, emission wavelength 512 nm; Molecular Probes), as described previously (Low and Heinsteins, 1986; Apostol et al., 1989a). Basically, 1.5 mL of cell suspension was treated with pyranine and transferred to a spectrofluorimeter cuvette where it was maintained in suspension by mild stirring. Typically, the quantity of pyranine used was $7 \mu\text{L}$ of a 0.2 mg/mL stock solution in distilled water ($0.93 \mu\text{g/mL}$ final concentration) and elicitor was added when the stirring started. Several experiments, however, required the use of a different quantity of pyranine or employed varying amounts of stirring before elicitor addition. These changes have been mentioned in the figure legends.

Viability Stain

Ten milliliters of cell suspension were treated for 15 min with fluorescein diacetate (1 mg mL^{-1} of stock in DMSO; Molecular Probes) at a final concentration of $30 \mu\text{M}$. Extracellular fluorescence was then removed by washing the cells with 10 volumes of fresh medium and resuspending them in fresh culture medium. Dye loading by live cells was finally observed by fluorescence microscopy using the appropriate filter set (excitation filter, EY455; emission filter, G520, Olympus).

RESULTS

Analysis of the Fraction of Soybean Cells Participating in the Oxidative Burst

Although previous studies revealed that many fluorescent dyes were oxidatively modified by elicitor-stimulated soybean cells (Apostol et al., 1989a), we have selected pyranine for use in current assays because it is destroyed rapidly during elicitation, it is water soluble and membrane impermeable, and it is nontoxic to plant cells in culture. Its emission intensity is also pH insensitive below pH 6.5, i.e. the pH range of our soybean cell suspension (Clement and Gould, 1981). To evaluate the percentage of cells producing H_2O_2 during elicitation, we transferred a pyranine-containing cell suspension immediately following elicitor addition to a microscope slide and monitored the quenching of fluorescence in a field of cells by fluorescence microscopy.

The micrographs shown in Figure 1 display three representative time points during the elicitation period (A–C). During the 1st min following PGA addition, the pyranine

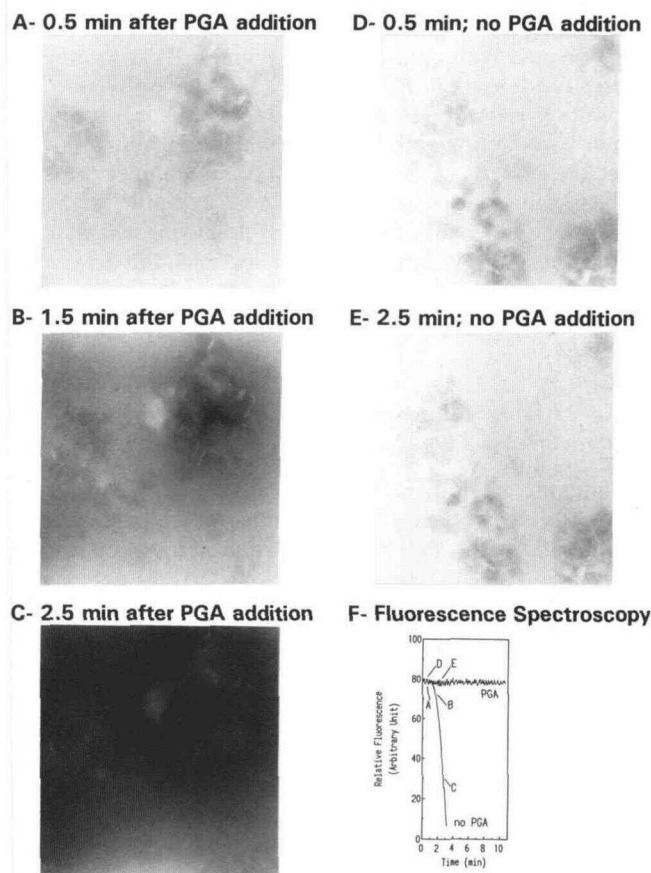


Figure 1. Observation by fluorescence microscopy of the time course of the PGA-induced oxidative burst. A soybean cell suspension (0.5 mL) was mixed for 15 s with 1.4 μg of the fluorescent dye pyranine (2.8 $\mu\text{g mL}^{-1}$ final concentration) and 6.78 μg of PGA (13.5 $\mu\text{g mL}^{-1}$ final concentration). An aliquot was subsequently placed on a microscope slide, and the fluorescence pattern observed by fluorescence microscopy. Pictures were taken every minute starting 30 s after PGA addition (A–C) or addition of water as a control (D and E) using the same exposure time for each photograph, as described in "Materials and Methods." Autofluorescence of the cells prevents the cells from appearing completely dark by the 2.5-min time. The kinetics of this elicitation event were also examined using a macroscopic assay (F) carried out on the same cell suspension. For this purpose, 1.5 mL of cells initially treated with the same elicitor and fluorescent reporter dye concentrations as described above were maintained in suspension in a spectrofluorimeter while fluorescence changes were recorded with time. The letters indicated on the PGA-induced (A, B, and C) and control (D and E) curves of F correspond to the times when the micrographs in A through E were taken.

fluorescence was uniformly distributed in the growth medium surrounding the field of cells, as shown at the 30-s time point of Figure 1A. However, by 1 min 30 s postelicitation, a dark periphery of bleached pyranine had begun to appear around most cells and cell clusters (Fig. 1B). In certain pockets, where diffusion was limited and H_2O_2 concentrations could increase rapidly, pyranine fluorescence was totally destroyed by this time point. Within the next minute, essentially 95% of the plated cells were enclosed in a blackened periphery

(Fig. 1C), and by 3.5 min postelicitation, the quenched area had expanded concentrically around each cell/cell cluster leaving the entire field dark, so that the reaction could no longer be followed. Cells to which no PGA had been added remained brightly fluorescent for the entire time (Fig. 1, D and E).

Importantly, the quenching kinetics seen in this assay were essentially identical with those observed by fluorescence spectroscopy (Fig. 1F) on the same cell suspension containing the same pyranine concentration, suggesting that the manipulations required for microscopy did not measurably perturb the assay. Because the quenching reaction is catalyzed by plant peroxidases present in the cell wall and suspension medium, we assume the soybean-generated H_2O_2 caused the oxidative destruction of the pyranine as it diffused radially from the cells into the adjacent solution. The fact that approximately 95% of the cells also scored positively in a viability stain (data not shown) suggests that essentially every living cell in the suspension engages in the elicitor-induced oxidative burst. Although the H_2O_2 could freely dilute into the entire suspension volume, H_2O_2 generated in vivo would likely be concentrated near its site of elicitation, allowing localized levels near the pathogen to increase precipitously.

Intensity and Duration of the Oxidative Burst

Although the oxidative destruction of pyranine by elicited plant suspension cultures has been well documented for several cell lines (Low and Heinsteins, 1986; Apostol et al., 1989a, 1989b), it has never been quantitatively characterized with a purified elicitor. The data of Figure 2 provide such characterization for soybean cells elicited with PGA (degree of polymerization approximately 13–14, as estimated by gel filtration chromatography using Bio-Gel P-6DG) purified according to the method of Nothnagel et al. (1983). As shown in Figure 2A, increasing concentrations of PGA promoted an increase in both the magnitude and rate of dye quenching. In contrast to the effect of a crude elicitor from the fungal pathogen *Verticillium dahliae* (Low and Heinsteins, 1986), greater quantities of elicitor did not lead to decreases in lag time before H_2O_2 release. The extent of dye quenching was observed to saturate near PGA concentrations of 2.5 $\mu\text{g mL}^{-1}$ (Fig. 2), and no further quenching of pyranine was measured even after addition of 40 $\mu\text{g mL}^{-1}$ of elicitor (data not shown).

A calibration curve relating the quantity of pyranine oxidized to the quantity of H_2O_2 released into the medium was constructed by adding varying amounts of exogenous H_2O_2 to a stirred cuvette containing an unelicited soybean cell suspension and measuring the decrease in fluorescence in a fluorescence spectrophotometer (data not shown). Although this calibration curve was found to vary by $\pm 30\%$ among different batches of cells, it was highly reproducible ($\pm 5\%$) within the same batch of cells. From the calibration curve constructed with the cells of Figure 2, it could be determined that addition of approximately four molecules of H_2O_2 leads to the quenching of one molecule of pyranine under our assay conditions (presumably three of the four molecules of H_2O_2 were consumed or detoxified in other reactions).

Based on this conversion factor and the data in Figure 2 (tracing obtained with 1.81 $\mu\text{g mL}^{-1}$ of PGA), we estimate

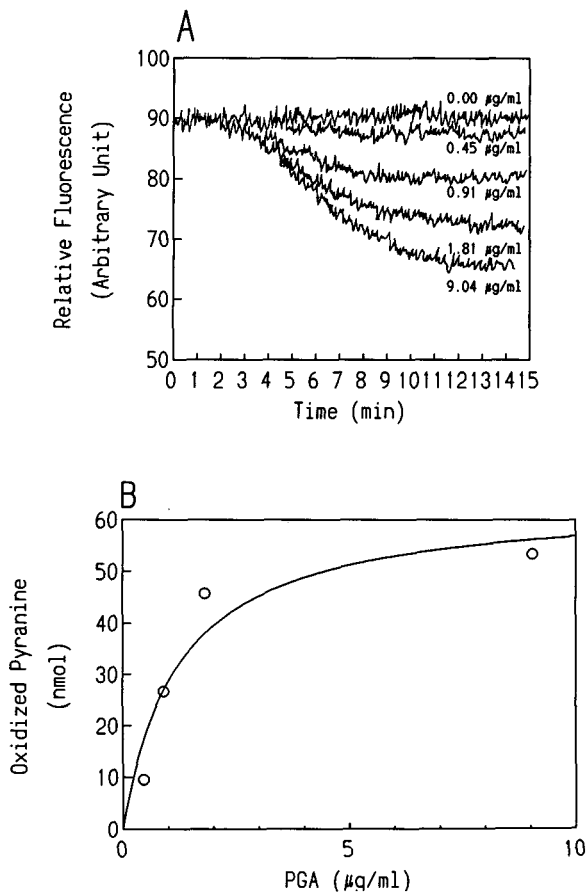


Figure 2. Kinetics of the PGA-induced oxidative burst. A stirred soybean cell suspension (1.5 mL) was treated with 50 μg of the fluorescent dye pyranine ($33.3 \mu\text{g mL}^{-1}$ final concentration) and with varying concentrations of PGA, as indicated on the graph. Each sample was then slowly stirred in the spectrofluorimeter chamber where fluorescence changes were recorded with time (A). Because each fluorescence transition proceeds from an initial fluorescence intensity to a subsequent stable value, it was possible to calculate the total quantity of dye that had oxidized upon addition of each quantity of PGA (B). This value is proportional to the amount of H_2O_2 that has been produced during an oxidative burst. It should be noted that the pyranine concentrations in this study ($33.3 \mu\text{g mL}^{-1}$) were more than 10-fold the concentrations used in Figure 1. Thus, for the same decrease in relative fluorescence, 11.9-fold more dye was bleached in Figure 2 than in Figure 1.

that 1 mL of the elicited soybean cell suspension releases approximately $0.12 \mu\text{mol}$ of H_2O_2 . Assuming that all of the H_2O_2 generated is freely accessible to extracellular peroxidases (an assumption that can only lead to an underestimate of the quantity of H_2O_2 produced), this amounts to approximately 100 molecules of H_2O_2 for each molecule of PGA added. Because the packed cell volume of the cell suspension was 10% of the total volume, had the cells not been diluted in growth medium the total concentration of H_2O_2 released would have amounted to approximately 1.2 mM. Because no quantitative information is yet available concerning H_2O_2 production in intact plants, we offer this value as a possible order of magnitude estimate of H_2O_2 production at the site

of elicitation *in vivo*, although we recognize that the estimate was obtained with optimally responding cells at saturating elicitor concentration. The value is still noteworthy because the same estimate has been provided for granulocyte production of H_2O_2 during pathogen inactivation in mammals (Morel et al., 1991). Moreover, on a cell basis, granulocytes and soybean cells generate H_2O_2 at a rate of 1×10^{-14} and $3 \times 10^{-14} \text{ mol cell}^{-1} \text{ min}^{-1}$, respectively, following stimulation with pathogen (calculated from data in Fig. 2 and data from Morel et al., 1991). Thus, the plant cell may be as well prepared to fight infection as disease-fighting cells in mammals.

Major Variables Influencing the Properties of the Oxidative Burst

Numerous stimuli modulate the response of a plant to pathogens *in vivo* (Bell, 1981), and three variables have been found to be particularly potent in controlling the rate and magnitude of H_2O_2 production *in vitro*. First, as noted previously (Apostol et al., 1989b), cell culture aging leads to a gradual diminution of the oxidative burst. When the quantitative analyses described above is used, half-maximal response to PGA was found to shift from $0.66 \mu\text{g mL}^{-1}$ of PGA for soybean cells 24 h after transfer to new medium to $6.6 \mu\text{g mL}^{-1}$ 60 h after transfer. During this same period, the lag time preceding the start of H_2O_2 production increased from 1 to 4 min, and the total duration of H_2O_2 biosynthesis decreased from 15 to 2 min. Clearly, all aspects of the oxidative burst are seen to attenuate with cell age. For reproducible results, cell cultures were invariably assayed 24 to 36 h after subculturing.

Mechanical stress, a variable known to have an impact on defense responses *in vivo*, was also observed to modulate H_2O_2 release in suspension-cultured cells (Apostol et al., 1989a). During the first 24 h after subculturing, soybean cells were frequently observed to constitutively generate low amounts of H_2O_2 , probably as a consequence of the shift to fresh medium. However, by 24 h posttransfer, most cell cultures no longer released these unstimulated oxidants but still produced significant amounts of H_2O_2 simply as a result of vigorous mechanical stirring. Although reducing stirring rate to a speed barely capable of maintaining the cells in suspension often alleviated the autoelicitation, older cells (approximately 36 h old) were generally used to avoid the elicitor-independent response, and elicitor-free controls were invariably conducted to assure that no elicitor-independent dye quenching occurred during at least the first 20 min of continuous stirring.

Even when mechanical elicitation was eliminated by selecting an older population of cells, excessive mechanical stress was found to modulate the oxidative burst. Thus, when cells insensitive to normal stirring speeds were continuously agitated at high speed for an extended period (approximately 10 min), a gradual diminution of the subsequent elicitor-stimulated response was observed, and this decrease in sensitivity became increasingly intense during the course of a day's experiments (Table I). Still, the magnitude of this desensitization was insufficient to explain cessation of H_2O_2 synthesis at the end of a normal oxidative burst. Furthermore,

Table 1. Effect of mechanical stress on the PGA-induced oxidative burst

Soybean cells (1.5 mL) treated with pyranine ($0.93 \mu\text{g mL}^{-1}$) were stirred vigorously for 10 min in the spectrofluorimeter. PGA ($10.17 \mu\text{g}$; $6.78 \mu\text{g/mL}^{-1}$ final concentration) was then added, and the elicited response evaluated as described in Figure 3 legend was compared to the value obtained if no stirring had occurred (percentage of desensitization). This experiment was then repeated several times during the same day on the same cell suspension to evaluate the effect of plant cell aging on the stirring-induced loss of sensitivity. Similar effects were observed in three other experiments.

Time after First Assay	Desensitization
min	%
0	19
40	56
80	81
110	87
160	88

use of the slowest stirring speed required for complete cell mixing and initiation of the assay soon after transfer to the stirred cuvette generally eliminated all stirring artifacts.

Although the soybean suspension cultures we use do not undergo intracellular or extracellular pH changes during elicitation (Horn et al., 1992), the cells are, nevertheless, very sensitive to medium pH in their oxidative burst. As shown in Figure 3, increasing the suspension pH with NaOH by 0.8 pH unit directly before elicitor addition increases the magnitude of the fluorescence transition, whereas decreasing the pH with HCl by 1.2 pH units induced the opposite response. Because pyranine is pH insensitive below pH 6.5 (Clement and Gould, 1981), and because there is no endogenous pH change during this time interval (Horn et al. 1992), the observed pH effects must derive from the pH sensitivity of some plant component involved in pyranine oxidation. Although the peroxidases that catalyze dye oxidation are pH dependent, pH modulation of their activity is insufficient to explain the magnitude of the effects in Figure 3. Therefore, we suggest that the H_2O_2 -generating mechanism in the plant is also subject to pH modulation. We believe that this observation is important, because hypersensitivity or hyposensitivity to elicitation could in some cases be promoted by a shift in extracellular pH. Furthermore, in studies of cultured cells, failure to adjust the pH of the added elicitor solution to the pH of the cell suspension could lead to variability in the data. Other perturbations of culture conditions that result in abnormal pH regulation could also lead to inconsistencies in the oxidative burst.

Desensitization of the PGA-Induced Oxidative Burst

The diminished response of a cell following a second exposure to the same stimulus (homologous desensitization) or different stimulus (heterologous desensitization) can protect a cell against the frequently deleterious effects of overstimulation (Sibley and Lefkowitz, 1985). Although it is a common property of signal transduction pathways in animal

cells, to our knowledge desensitization has only been implicated once in plants (Campbell and Labavitch, 1991). To learn whether homologous desensitization might be a characteristic of the PGA-induced oxidative burst, the magnitude of a second elicitation with saturating PGA was examined as a function of the strength of the initial stimulation. As displayed in Figure 4, very low levels of initial elicitation indeed disproportionately reduced the ability of the cells to respond to a second stimulus. Thus, when the soybean culture was initially induced to generate H_2O_2 at 2% of its maximal capacity, stimulation 10 min later with saturating PGA produced only 50% of the anticipated burst. Possible receptor occupancy cannot explain the diminished response, because presumably at most 2% of the sites were ligated with PGA during initial stimulation. Instead, we suggest the failure to respond to a second elicitation derived from a temporary inactivation of the signaling pathway, possibly through a feedback loop involving receptor phosphorylation or G protein ADP-ribosylation (Legendre et al., 1992).

To confirm that simple competition for putative PGA receptors did not cause the desensitization seen at low PGA concentrations, an independent inducer of the oxidative

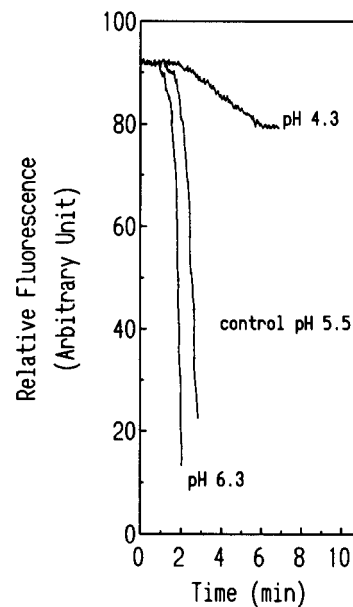


Figure 3. Effect of altered external pH on the PGA-induced oxidative burst. A stirred soybean cell suspension (1.5 mL) was initially treated with 7 μL of the fluorescent dye pyranine ($0.93 \mu\text{g mL}^{-1}$ final concentration) either alone (control) or in combination with 10 μL of NaOH (0.1 N stock) or 15 μL HCl (0.1 N stock). After 4 min of equilibration, 5 μL of PGA ($2.26 \mu\text{g mL}^{-1}$ final concentration) was added to each of the three samples, and their fluorescence intensity was readjusted to a similar level. Subsequent fluorescence changes were then recorded, starting at the time of elicitor addition. The equilibrium pH of each of these samples was determined by placing a pH electrode into each cell suspension. Values were pH 5.5 for the control cell suspension, pH 6.3 for the NaOH-treated cell suspension, and pH 4.3 for the HCl-treated cell suspension. A low dye concentration was used to allow the low pH inhibition to be more easily visualized. Similar results were obtained in a separate experiment.

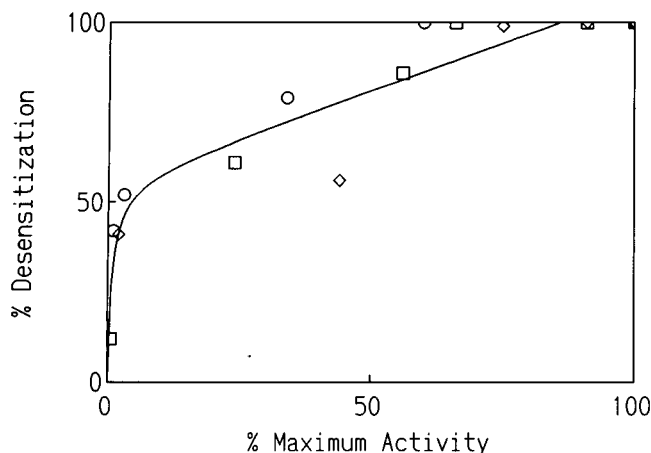


Figure 4. Loss of sensitivity to PGA after pretreatment with low concentrations of the same elicitor. A soybean cell suspension (1.5 mL) was treated with the fluorescent peroxidase substrate pyranine ($0.93 \mu\text{g mL}^{-1}$ final concentration) and varying concentrations of PGA. The fluorescence intensity of the sample was then recorded as described in "Materials and Methods," except the total fluorescence was maintained between 30 and 90% of its original value by regular additions of fresh pyranine. Ten minutes later, when dye oxidation had ceased, the fluorescence level was readjusted to its original value with fresh dye, and the total amount of pyranine that had been added was evaluated. The same cells were then rechallenged with a saturating PGA concentration ($6.78 \mu\text{g mL}^{-1}$ final concentration), and the new quantity of oxidized pyranine was determined. Values on the x axis indicate the intensity of the initial stimulation as a percentage of the maximum possible stimulation, i.e. that obtained upon treatment with $6.78 \mu\text{g mL}^{-1}$ of PGA (saturating amount). Values on the y axis (percentage of desensitization) compare the quantity of pyranine that is oxidized during the second stimulation with the quantity that would have been oxidized in the absence of prior elicitation. This experiment was conducted three times (O, experiment 1; ◇, experiment 2; □, experiment 3).

burst, the peptide mastoparan (Legendre et al., 1992) was substituted for PGA as the initial stimulant. Importantly, mastoparan, but not its inactive homolog, Mas17 (Higashijima et al., 1990), was also able to desensitize the soybean cells to PGA (Fig. 5). Because mastoparan directly activates intracellular G proteins (Higashijima et al., 1988) presumably without competing for PGA receptors, its impact on PGA elicitation should not involve obstruction of available cell-surface receptors. Rather, an autoregulatory loop in the signal transduction pathway is likely involved. Peculiarly, the mastoparan, which at maximal strength is a much weaker elicitor than PGA (Legendre et al., 1992), was able to desensitize the cells much more rapidly than PGA, i.e. within only 3 to 4 min (Fig. 5).

DISCUSSION

In previous studies of the oxidative burst in cultured plant cells, the use of crude elicitor preparations prohibited a quantitative evaluation of the various properties of the defense response. However, use of the pure pectic oligomer PGA has now allowed us to characterize the defense response

more definitively. We conclude that essentially all cells in a suspension participate in a rapid oxidative burst, producing an H_2O_2 concentration of approximately 1.2 mmol L^{-1} of packed cells (approximately 100 molecules of H_2O_2 molecule $^{-1}$ of PGA added) at a rate of $3 \times 10^{-14} \text{ mol of H}_2\text{O}_2 \text{ cell}^{-1} \text{ min}^{-1}$ in a manner that is saturably dependent on elicitor concentration. Although several variables were identified that can alter both the quantitative and qualitative aspects of the burst, when care is taken to control for cell age, cell suspension pH, mechanical stimulation, and premature desensitization, highly reproducible results can be obtained. Thus, with the aid of a saturation plot such as that shown in Fig. 2B, the effects of suppressors and enhancers of elicitation can be quantitatively defined in terms of their effects on the apparent K_D and V_{max} of the elicitor-induced response.

In addition to providing quantitative information, the current study permits a comparison of the properties of the oxidative burst elicited with PGA with that elicited by a crude extract from the phytopathogenic fungus *V. dahliae*. Whereas the lag time between elicitor addition and onset of H_2O_2 production was independent of PGA concentration, elevated concentrations of the fungal extract resulted invariably in shorter lag times (Low and Heinsteins, 1986). Furthermore, whereas the rate of H_2O_2 release (slope of quenching curve in Fig. 2A) monotonically increased with increasing PGA concentrations, the same parameter increased to a maximum and then declined in the case of the *V. dahliae* elicitor. Finally, although not previously reported, low concentrations of fungal extract do not invariably lead to low cumulative levels of H_2O_2 production. Instead, with many crude fungal extracts,

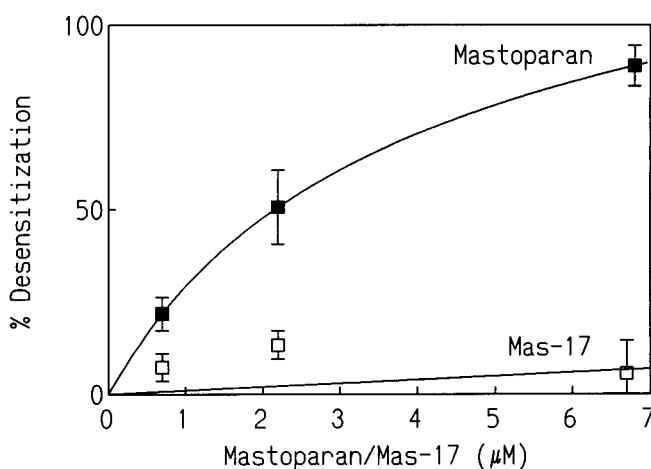


Figure 5. Loss of sensitivity to PGA following pretreatment with mastoparan or Mas-17. A soybean cell culture (1.5 mL) was first treated with $0.93 \mu\text{g mL}^{-1}$ of a fluorescent dye (pyranine) and varying concentrations of mastoparan or Mas-17. Three to four minutes after this first stimulation, because the mastoparan-induced oxidative burst had stopped, a saturating concentration of PGA ($6.78 \mu\text{g mL}^{-1}$ final concentration) was added to the cells. This second elicitation response was determined as described in Figure 3 and compared to the one obtained with cells not prechallenged with any stimulant (percentage of desensitization). The plot shown represents the average of three separate experiments.

oxidant production simply begins after a longer delay and accelerates slowly until high levels of H_2O_2 are ultimately generated. Clearly, unidentified components of the fungal elicitor preparation lead to a more complex response than that encountered with pure PGA. Recent data from our laboratory demonstrate that the fungal extract actually contains several elicitors, a protein component that stimulates phytoalexin but not H_2O_2 production, and other lower mol wt fractions that elicit exclusively the oxidative burst (Davis et al., 1993). Cross-talk between the several signal transduction pathways could readily modulate the net response.

As noted above, younger cells can often be mechanically stimulated to produce H_2O_2 in the absence of elicitor. We do not believe this stimulation arises from mechanical fragmentation of the cell wall with its consequent release of pectic oligomers, because in many cases even the mildest possible agitation can initiate H_2O_2 biosynthesis. Rather, we speculate that the mechanical induction of H_2O_2 in rapidly growing but not senescent cells may be more involved in processes related to thigmotropism than elicitation. Thus, sites of mechanical stress in a growing plant are frequently stabilized by deposition of additional extracellular matrix/cell wall components, and these deposition processes often require H_2O_2 (Mäder and Amberg-Fisher, 1982; Bradley et al., 1992). It would be interesting to learn whether the same oxidase complex is used in both elicitation and thigmotropism pathways.

The production of large quantities of H_2O_2 on the surface of a cell has the potential to cause extensive cellular damage if not properly controlled. In myeloid cells of animals and also during egg fertilization, a series of second-messenger pathways control the quantity of oxidant released and assure that its production is terminated when it is no longer needed (Morel et al., 1991; Shapiro, 1991). Data presented here indicate that oxidant production in soybean cells is also subject to regulatory controls. First, PGA-induced oxidant production is both measured in quantity and finite in duration. Furthermore, after an initial stimulation with elicitor, the cells become desensitized to subsequent stimulation, possibly to avoid further H_2O_2 release and the consequent oxidant damage. That this response is under second-messenger control is evidenced by the ability of mastoparan, which has been shown to activate G proteins involved in soybean elicitation (Legendre et al., 1992), also to desensitize the system. Indeed, the gradual loss in elicitor sensitivity of older cells following extended high-speed stirring could also derive from a similar regulatory pathway.

Our estimate that elicited soybean cells can generate a localized H_2O_2 concentration of approximately 1.2 mM indicates that the oxidative burst is likely sufficiently intense to be cytotoxic to invading pathogens. Similar H_2O_2 concentrations were identified by Kim et al. (1988) as the primary antibiotic activity in the culture filtrate of *Talaromyces flavus*, and analogous oxidant concentrations are released by neutrophils in their defense against pathogens in mammals (Morel et al., 1991).

Based on this concept, we wish to offer a hypothesis explaining the role of the oxidative burst in total plant defense. As a pathogen begins to colonize a host plant cell, cell wall digestion products (e.g. PGA) and other pathogen-de-

rived molecules may be released into the plant tissue. These elicitors and pectic fragments may subsequently be recognized by plant cell surface receptors, leading to the rapid production of O_2^- and H_2O_2 . Although the localized oxidant concentration may increase significantly, because the pectic oligomers do not diffuse from the site of production (Baydoun and Fry, 1985) and because plant cells are equipped with extensive antioxidant activities (i.e. catalases, superoxide dismutases, peroxidases, glutathione), the H_2O_2 burst will be confined largely to the site of pathogen attack. By this means, the invading pathogen (and surrounding host plant cells) may be rapidly damaged or destroyed, allowing adjacent cells time to mount a more diversified defense response. Where H_2O_2 is able to escape its immediate site of synthesis, it may also contribute to pathogen resistance, because H_2O_2 can aid in cell wall cross-linking/lignification reactions (Bradley et al., 1992) and promote phytoalexin production (Apostol et al., 1989a; Montillet and Degoussee, 1991; Sharma and Mehdy, 1992). Presumably, with the added preparation time provided by the rapid, localized assault with oxidants, the remainder of the plant can often mobilize its slower but more systemic defenses to allow successful resistance to the pathogen.

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